needed to be included does exist. I am still at a loss as to why Kemin failed to include such information long ago.

Response to Objections to Claims

1. As the primary inventor and author of the patent, I made every attempt to meet the spirit of the first paragraph of 35 U.S.C. 112. It was written in such a way as to ensure that those skilled in the art would be able to replicate the findings of my research. The methodology described in the protocols of the patent is standardized as to the search for such bacteria that possess the ability to produce antimicrobial compounds. Madigan et al. state "The classical procedure for testing new microbial isolates for antibiotic production is the cross streak method, first used by Fleming in his pioneering studies on penicillin." (Brock Biology of Microorganisms, 2003. 10^{th} edition, page 974) (see addendum). This streak method on newly isolated bacteria, regardless of the source, is well known and what I attempted to describe in the patent.
2. I in no way refute that the existence of microorganisms on and in organisms is certainly well known. My research focuses upon amphibians as the source of medically significant bacteria. Perhaps the title of the patent should have been more narrow, a suggestion that I made to the patent attorney. Never-the-less, I never intended to indicate that all bacterial isolates would possess the ability to produce antimicrobial compounds. Most simply won't. But I have effectively demonstrated that some will. Further, I never intended to imply through the title of the patent that specific compounds would be structurally illucidated. Again, the claims were written broadly to ensure that biologically active molecules extracted from source bacteria would be covered under the patent. I am providing an unpublished white paper that was written which describes the structure and antimicrobial activity of a new class of cyclic undecapeptides produced by a bacterium isolated from the skin of an amphibian. This paper describes the structure in intricate detail and, in my opinion, should have been included in our initial patent materials or in our first response to your rejection (see addendum). Additionally, I am including a copy of an email that indicates the molecular weight and chemical formula of another compound that was isolated from an extract produced by a bacterium which was isolated from the skin of an amphibian (see addendum).
3. I am also enclosing data for the 16S rRNA gene sequence analysis for five of the bacteria isolated from amphibians that exhibited the ability to inhibit known human pathogenic bacterial species (see addendum). This research was completed in February, 2003 and should have been made available to you. As you examine this information, please note that this type of analysis compares the 16S rRNA gene sequence to known bacterial species and gives a percent match. None of my research isolates gave a 100% match, indicating that there are at least strain differences between my isolates and the data bank to which they were compared. This data would give others the ability to compare their results to determine if their isolates were the same as mine. As to allowing access for others to my bacterial library, I would make them available to anyone who requested them.

That being said, I am simply not in the financial position to deposit my bacterial isolates in the ATCC depository bank. To do so would cost many thousands of dollars. Kemin knew this and also knew that by not providing the funds necessary to deposit said bacteria in ATCC would likely provide a major stumbling block to the perfection of this patent. As I stated earlier, I am an associate professor of biology at a small college in Georgia who makes barely enough to raise my family on. I am no longer in any partnership with any company. To require this as part of the patent requirements would be discriminatory. Please reconsider this part of your argument.

This patent application represents years of my personal research. It also successfully represents that amphibians are a novel source of bacteria that are capable of producing novel compounds that could be medically useful. Further, I have demonstrated that they are capable of inhibiting growth in bacteria, viruses and tumor cell lines.

As the world feverishly searches for answers for antibiotic-resistant bacterial infections, impending flu pandemics and never-ending sieges by cancer, this research, provides a more than plausible or possible answer for our world's needs. I have compounds isolated from bacteria found on amphibian skin that have shown great promise against diseases such as MRSA, VRE, and HIV to name a few. If this patent is ultimately rejected, my research ends...there isn't any more funding left and the world may never know what might have been available. It will also likely allow a major cooperation to bury this research so that they might use it for their own benefit in the future without having to give a portion of proceeds to someone else.

I am respectfully requesting that you please view this information included in this response as reconsider your earlier objections. If you see it in your heart to allow at least some of the claims, then I'll be able to pursue more funding. Better yet, I might be able to hand this off to a company that has the resources to work in a much more efficient and rapid manner than I could ever do as a full-time teaching professor. This isn't about money for me. This is about solving world problems through science. Please reconsider your objections to the claims. The world might hang in the balance...and yes, I believe in it that much. I just should have taken control of the responses to the patent earlier instead of letting a corporate-world attorney and major company handle it.

Please send all further communications to me personally. If I need to fill out any necessary paperwork, please send it as well. My address is as follows:

Dr. Rick Austin
Associate Professor of Biology
Piedmont College
165 Central Avenue
Demorest, GA 30535

I appreciate any and all reconsideration you can give in this matter. Please feel free to contact me should you have any questions at all.

Best Regards,

Rick Austin, Ph.D.

Dr. Rick M. Austin
(706) 778-3000 ext 1167

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2100 Maury Street
Des Moines, IA 50317-1100 USA
tel: 515.559.5100 fax: 515.559.5232
toll free: **800.777.8307**
www.Kemin.com

Our Vision:
We strive to improve the quality of life by touching half the people of the world every day with our products and services.

February 7, 2006

Received
Feb 10, 2006

Dr. Rick Austin
Plethodon Research LLC
1590 Double Springs Road
Demorest, Georgia 30535

RE: "Bacterial isolates from organisms that respire at least partially through their skin and biologically active extracts derived therefrom" (KEM 42)

Dear Dr. Austin:

As you know, Kemin Industries and Plethodon have terminated their joint research activities. We believe that the only action required of Kemin Industries is to return to Plethodon any biological materials it has received from Plethodon. We will gather these materials and await your instructions with regard either to destruction of the materials or the address to which you would like them delivered. Destruction of the materials makes the most sense to us since you have your own cultures of these materials. We would be happy to provide you with a letter certifying destruction of the cultures when that has occurred.

As a matter of courtesy, we remind you of the approaching deadline of February 11, 2006 for responding to the August 11, 2005, Office Action on the pending US patent application, referenced above. Given the termination of the relationship, you may elect to proceed with prosecution of the patent application independently. I am sure that Kent Herink would work with you or alternative legal counsel if there are any questions.

If you have any questions, please do not hesitate to contact me directly either by telephone at 515/559-5347 or by e-mail at libby.nelson@kemin.com.

Regards,
KEMIN INDUSTRIES, INC.

Elizabeth A. Nelson

Elizabeth A. Nelson
Corporate Counsel

cc: Benedikt Sas
Kent Herink
Andrew G. Yersin, Ph.D.

Identification Report Summary

Customer: Austin

2/28/2003

MIDI LABS

Full Length 16S rRNA Gene Sequence Analysis

MicroSeq Database

C #	sample	closest match	% difference	confidence level
N520AUS	C9857 DQ001D con	Pseudomonas tolaasii	0.03 %	Species
N520AUS	C9858 P0027 con	Pseudomonas fluorescens A (bt)	0.16 %	Species*
N520AUS	C9859 P0026 con	Pseudomonas tolaasii	0.59 %	Species*
N520AUS	C9860 AOJUV017 con	Serratia marcescens	0.16 %	Species
N520AUS	C9861 EB002 con	Brevibacillus brevis	0.39 %	Species*

Key:

C code - Customer number assigned by MIDI Labs.

sample - Sample number assigned by MIDI Labs, followed by name assigned by customer.

closest match - Closest match to sample when aligned in a pairwise manner against the MicroSeq Database.

% difference - Percent difference between the sample and the closest match.

confidence level - This indicates the level of identification; see Identification Report Summary for additional information.

For research use only

Alignment Report - 1500 BP Identification

MIDI LABS

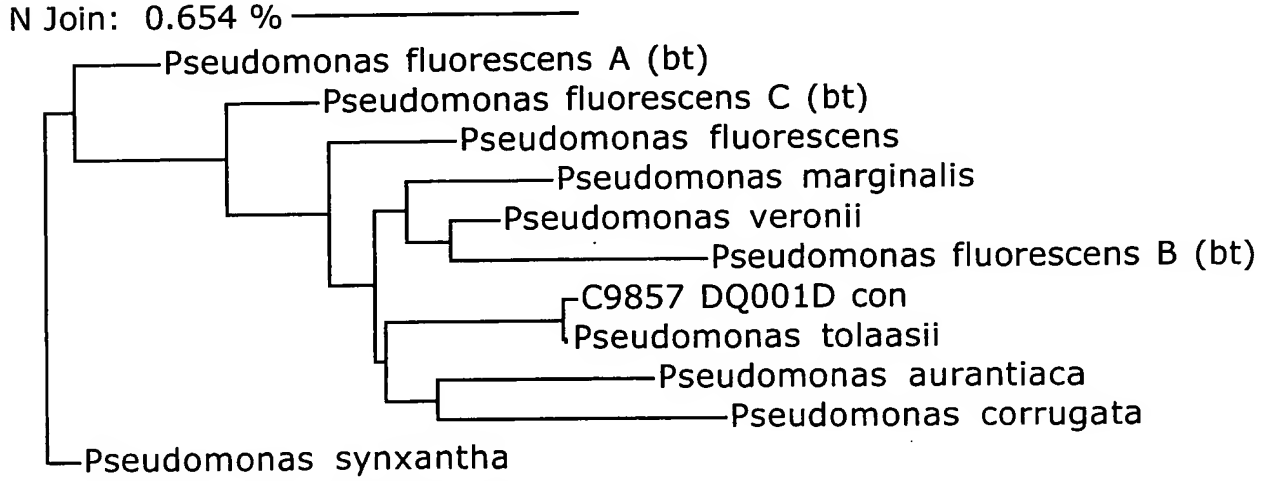
Customer: Austin

Sample: C9857 DQ001D con

Date: Friday, February 28, 2003 11:45 AM

Alignment: 1532 C9857 DQ001D con
0.03 % 1532 Pseudomonas tolaasii
0.72 % 1532 Pseudomonas veronii
0.85 % 1532 Pseudomonas marginalis
0.88 % 1532 Pseudomonas fluorescens
0.95 % 1532 Pseudomonas aurantiaca
1.01 % 1532 Pseudomonas fluorescens A (bt)
1.08 % 1532 Pseudomonas fluorescens C (bt)
1.24 % 1530 Pseudomonas fluorescens B (bt)
1.31 % 1532 Pseudomonas corrugata
1.34 % 1532 Pseudomonas synxantha

N. Joining Tree



Concise Alignment - 1500 bp

```
                          1
                          5
                          1
                          8
C9857 DQ001D con          Y
Pseudomonas tolaasii      C
```

Reviewer's signature

For Research Use Only.

MIDI LABS

Alignment Report - 1500 BP Identification

Customer: Austin

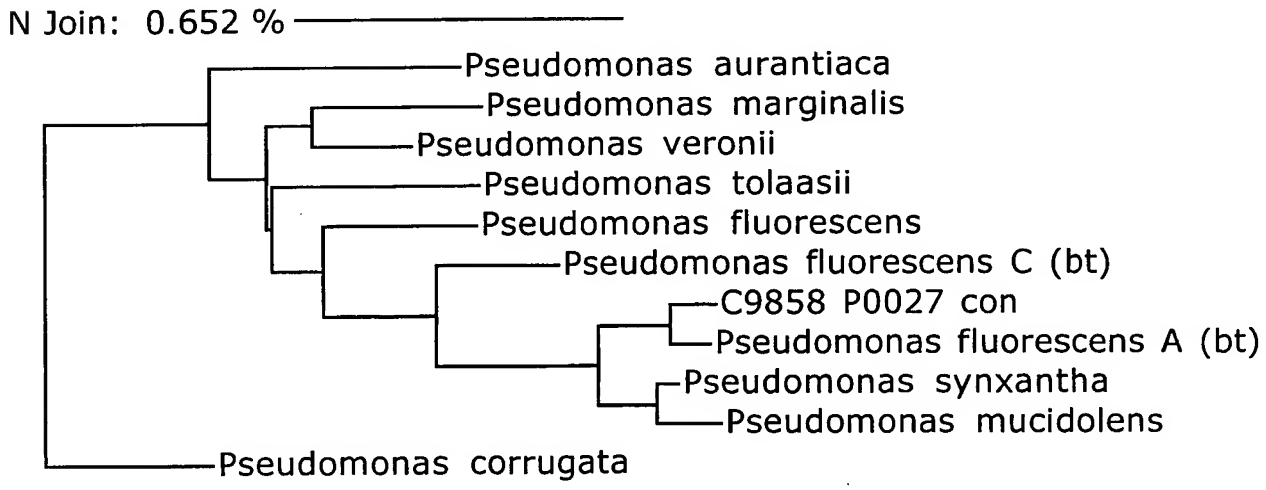
Sample: C9858 P0027 con

Date: Friday, February 28, 2003 11:46 AM

Alignment: 1532 C9858 P0027 con

0.16 % 1532 Pseudomonas fluorescens A (bt)
0.49 % 1532 Pseudomonas synxantha
0.52 % 1532 Pseudomonas mucidolens
0.88 % 1532 Pseudomonas tolaasii
1.01 % 1532 Pseudomonas fluorescens C (bt)
1.21 % 1532 Pseudomonas fluorescens
1.24 % 1532 Pseudomonas marginalis
1.24 % 1532 Pseudomonas veronii
1.40 % 1532 Pseudomonas aurantiaca
1.76 % 1532 Pseudomonas corrugata

N. Joining Tree



Concise Alignment - 1500 bp

```
                                  111111
                                  2788001225
                                  4323006471
                                  8679182278
C9858 P0027 con                   AAATCGAGTY
Pseudomonas fluorescens A (bt)    AAATTAAGTC
Pseudomonas synxantha             GGGCTAAGGC
Pseudomonas mucidolens            GGGCTARATC
```

Reviewer's signature

For Research Use Only.

This sample is closely related to P. fluorescens, P. synxantha, and P. mucidolens by 16S rRNA gene sequence.

Alignment Report - 1500 BP Identification

Customer: Austin

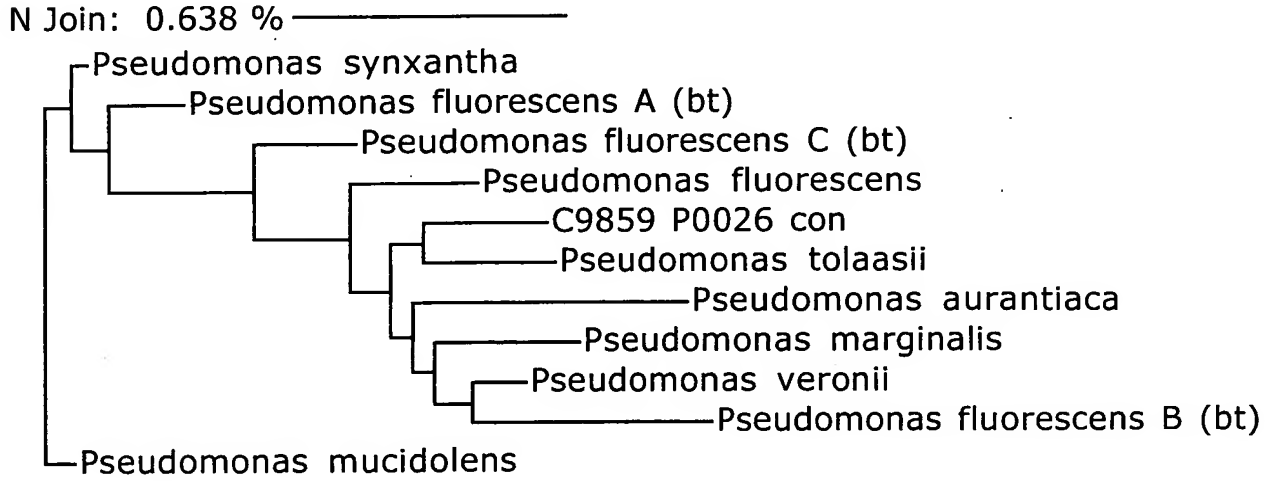
Sample: C9859 P0026 con

Date: Friday, February 28, 2003 11:47 AM

Alignment: 1532 C9859 P0026 con

0.59 % 1532 Pseudomonas tolaasii
0.65 % 1532 Pseudomonas fluorescens
0.69 % 1532 Pseudomonas veronii
0.82 % 1532 Pseudomonas marginalis
0.91 % 1532 Pseudomonas fluorescens A (bt)
0.98 % 1530 Pseudomonas fluorescens B (bt)
1.04 % 1532 Pseudomonas fluorescens C (bt)
1.17 % 1532 Pseudomonas aurantiaca
1.24 % 1532 Pseudomonas synxantha
1.27 % 1532 Pseudomonas mucidolens

N. Joining Tree



Concise Alignment - 1500 bp

```
                                  111111
                        1111444445788001245
                        1145455668323002741
                        1965801241679187778
C9859 P0026 con         TRTAGTAGTTAATTAATAY
Pseudomonas tolaasii    TACGGTGACTAATCGTTAC
Pseudomonas fluorescens AATGTAATGTAGCTAACAC
Pseudomonas veronii     TATGGTARTAGGCCGAGGC
```

Reviewer's signature

For Research Use Only.

This sample is closely related to P. tolaasii, P. fluorescens, and P. veronii by 16S rRNA gene sequence.

MIDI LABS

Alignment Report - 1500 BP Identification

Customer: Austin

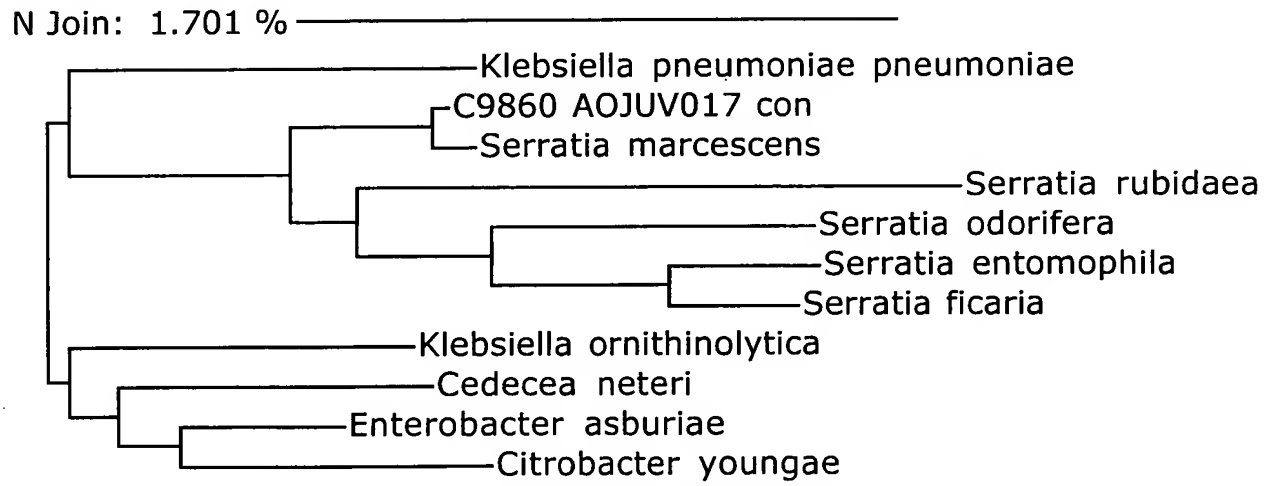
Sample: C9860 AOJUV017 con

Date: Friday, February 28, 2003 11:48 AM

Alignment: 1537 C9860 AOJUV017 con

0.16 % 1537 Serratia marcescens
1.85 % 1537 Serratia entomophila
1.99 % 1536 Serratia ficaria
1.99 % 1535 Enterobacter asburiae
2.05 % 1537 Serratia odorifera
2.18 % 1537 Cedecea neteri
2.18 % 1535 Klebsiella pneumoniae pneumoniae
2.28 % 1535 Klebsiella ornithinolytica
2.31 % 1537 Serratia rubidaea
2.33 % 1537 Citrobacter youngae

N. Joining Tree



Concise Alignment - 1500 bp

```
                          11
                        4422
                        5648
                        7709
C9860 AOJUV017 con      GCRY
Serratia marcescens     RTAT
```

Reviewer's signature

For Research Use Only.

Alignment Report - 1500 BP Identification

Customer: Austin

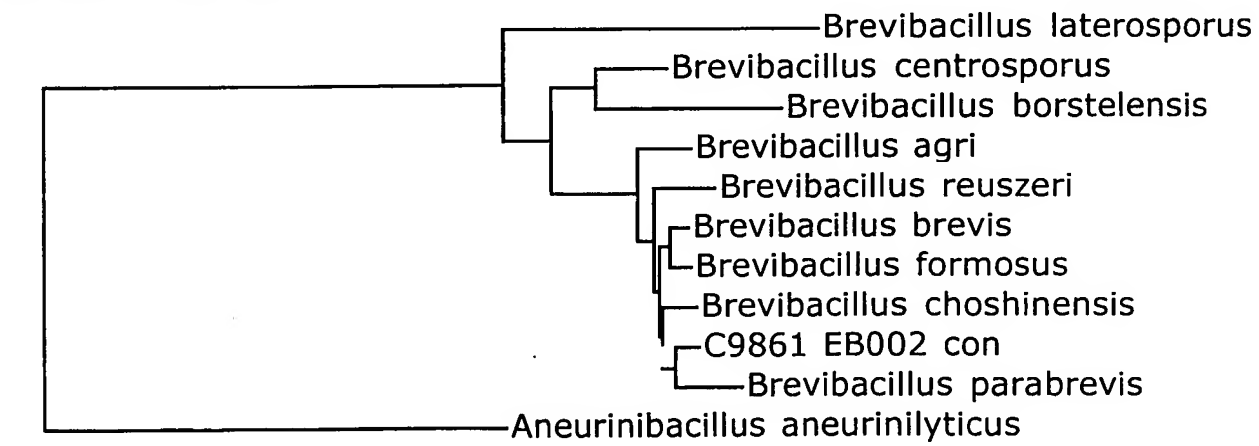
Sample: C9861 EB002 con

Date: Friday, February 28, 2003 11:50 AM

Alignment: 1531 C9861 EB002 con

0.39 % 1531 Brevibacillus brevis
0.56 % 1531 Brevibacillus choshinensis
0.72 % 1531 Brevibacillus formosus
0.75 % 1531 Brevibacillus parabrevis
0.95 % 1531 Brevibacillus reuszeri
1.31 % 1532 Brevibacillus agri
2.32 % 1531 Brevibacillus centrosporus
3.72 % 1531 Brevibacillus borstelensis
4.41 % 1531 Brevibacillus laterosporus
9.70 % 1534 Aneurinibacillus aneurinilyticus

N. Joining Tree



Concise Alignment - 1500 bp

```
                                1111
                        11111244661125
                    6677723689034011161
                    7867862925733750217
C9861 EB002 con             GGACCYAYCGGTTATCTGY
Brevibacillus brevis        TSACSTATCGGTTGCCTAY
Brevibacillus choshinensis  TCRGGTATCGGTCATTCGY
Brevibacillus formosus      TCAGGTRTYRRYTGCCTAC
```

Reviewer's signature

For Research Use Only.

ISOLATION, STRUCTURE ELUCIDATION AND ANTIMICROBIAL ACTIVITY OF A NEW CLASS OF CYCLIC UNDECAPEPTIDES DERIVED FROM BACTERIA LIVING ON THE SKIN OF AMPHIBIANS

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Kemin Pharma Europe
Atealaan 4 H, 2200 Herentals, Belgium

Austin, R.
Plethodon LLC
1590, Double Springs Road, Demorest, Georgia 30535, USA

ABSTRACT

Research in the antimicrobial area is recently driven by the discovery of new classes of antibiotics, due to an increasing resistance of micro-organisms to known anti-infectives. We describe the isolation and structure elucidation of a new class of cyclic undecapeptides, which are secondary metabolites of bacteria living in symbiosis on the skin of amphibians. Depending on the amino acid sequence, they show good antibacterial or antifungal or antiviral activity.

Keywords: antimicrobial activity, cyclic peptides, isolation, structure elucidation, amino acid sequence

INTRODUCTION

The increasing resistance to known antibiotics resulted in intensely pursued research into the development of new classes of antibiotics. This search for new molecules has led to the study of naturally occurring antimicrobial peptides as a foundation on which to target the lysis of micro-organisms by mechanisms that do not aid in the proliferation of resistant strains[1,2,3].

The widespread distribution of the molecules indicate that antimicrobial peptides have played a fundamental role in the successful evolution of complex multi-cellular organisms[4].

These peptides have been found in living organisms as such (plants, insects, animals and humans), but they also occur as secondary metabolites from bacteria that live in symbiosis with their host e.g. laxaphycin that is produced by the cyanobacteria *Lyngbya majuscula*[5].

Several peptides have been isolated from amphibian skin secretions[6].

Here we describe a new class of cyclic undecapeptides that are produced by gram negative rod bacteria which live in symbiosis with amphibians in the muscous layer of their skin. These peptides exhibit antibacterial, antifungal and antiviral activity. Depending on the specific amino acid sequence of the peptides, some selectivity in the antimicrobial properties can be observed.

METHODS AND MATERIALS

Study of the biological activity

Bacteria[7]

Antimicrobial activity and MICs were determined by a micro-dilution procedure using a Bioscreen (Labsystems, Finland) which is a robotic microbial growth analyzer. It monitors the growth of micro-organisms by measuring the change in optical density of the broth culture. White light (Wide Band) was used with no selection of any specific wavelength. The Bioscreen was controlled via a PC and the data capture software, Biolink. The measurement-parameters for analysis, such as incubation time and temperature, were determined for the particular experiment. Bacterial strains were obtained from the Belgian Co-ordinated Collections of Micro-organisms. The bacteria were grown in Mueller-Hinton broth (Oxoid, CM 405).

In order to create an anaerobic environment for the screening of *C. perfringens*, the Bioscreen analyser was inserted in a plexi-glass chamber. This chamber was purged with CO_2 (Air Liquide) in order to create an environment with minimal O_2 concentrations.

The bacterial strain *C. perfringens* ATCC 13124, was obtained from Oxoid as a Culti-Loop culture. *C. perfringens* was grown in Anaerobe Basal broth (Oxoid, CM 957).

From a 18 hour broth culture, instant suspensions of all the micro-organism were prepared in 5ml quantities of sterile saline. Inoculum was standardized according to the 0.5 McFarland standard and further diluted until a final inoculum of approximately 5×10^5 CFU/ml was obtained.

The test chemicals were new antimicrobial cyclic peptides (KPE01001001/0.1.0; KPE01001006/1.0; KPE01001007/1.3). Vancomycin (Fluka/BioChemika 94747), gentamycin (Fluka/Biochemika 48760) were used as reference antibiotics. All test chemicals were dissolved in DMSO in order to obtain a stock solution of 2500 ppm. Further 10-fold dilutions were made in a mixture of 11 parts demineralised water and 1 part DMSO.

Each of the 100 well honeycomb plates (Labsystems) contained 225 µl Mueller-Hinton broth and 25 µl of the solvent (negative control), test-chemical dilution, or positive control. The inoculum was added to the broth, except for the blanco tests. For the bacteria the incubation time was 16 hours and the incubation temperature was 35°C, the time between OD measurement was 20 minutes, with a medium shaking before each measurement.

Yeasts[8]

Two reference strains with known susceptibility patterns were purchased from the Belgian Co-ordinated Collection of Micro-organisms: *Candida albicans* IHEM 10248 and *Cryptococcus neofromans* IHEM 9558. A synthetic medium is recommended for fungal susceptibility tests RPMI 1640 broth supplemented with 0.3g/l glutamine and 34.6g/l 3-(N)-morpholino propane sulphonic acid (MOPS-buffer) without bicarbonate and with a pH indicator. All organisms should be sub-cultered from stock cultures onto Sabouraud dextrose agar and passaged at least twice to ensure purity and viability. The incubation temperature throughout must

be 35°C. The inoculum should be prepared by picking 5 colonies of ≥ 1 mm in diameter from 24 hour-old cultures of albicans or 48 hour-old cultures of *C. neoformans*. The colonies should be suspended in 5 ml of sterile 0.85% saline. The resulting suspension should be vortexed for 15 seconds and the cell density adjusted with a spectrophotometer by adding sufficient sterile saline to increase transmittance to that produced by a 0.5 McFarland standard at 530nm. This procedure yields a yeast stock suspension of 1 x 10^6 to 5 x 10^6 cells per ml. Further dilutions are made so a final inoculum of 1 – 5 x 10^3 CFU/ml is obtained in each well. The reference antimycotic amphotericin B was purchased from Fluka/Biochemika. The incubation time was 24 hours for *Candida albicans* and 48 hours for *C. neoformans*. The incubation temperature was 35°C.

The area under the growth curve for each of the different wells was chosen as critical parameter for the evaluation of possible antimicrobial and anti-yeast activity. Each test was done in 5 replicates and the average and the coefficient of variation were determined. Blanco area was subtracted from the sample area to obtain a delta area. The delta area of the negative control was set a 100% growth. Other % of growth were calculated relatively to the negative control.

Moulds[9]

Two reference strains were purchased from the Belgian Co-ordinated Collection of Micro-organisms: *Trichophyton mentagrophytes* IHEM 10342/ATCC 9533 and *Microsporum gypseum* IHEM 3999/ATCC 14683.

The inoculum is prepared by covering a seven days old culture with approximately 1 ml of sterile 0.85% saline. A suspension is made by gently probing the colonies with the tip of a Pasteur pipette. Addition of one drop of Tween 20 will facilitate the preparation of the inocula. The resulting mixture of spores and hyphal fragments is withdrawn and transferred to a sterile tube. After heavy particles are allowed to settle for 3 to 5 minutes, the upper homogenious suspension is collected and mixed with a vortex mixer for about 15 seconds. The densities of the spore-suspensions are read and adjusted to an optical density of 75% of transmittance at 530nm. The inoculum suspension should be diluted in RPMI 1640 broth, in order to obtain the inoculum size of 0.4 x 10^4 to 5 x 10^4 CFU/ml.

Anti-mould screening are also conducted in RPMI 1640 broth supplemented with 0.3g/l glutamine and 34.6g/l 3-(N)-morpholino propane sulphonic acid (MOPS-buffer) without bicarbonate and with a pH indicator. The dermatophytes have an incubation temperature of 25°C and the incubation time is 5 days.

For the moulds kinetically measurement was not used, but instead begin and end point measurement was performed. As measurement tool we use the OD (start and end), which is automatically determined via the Biolink software. OD-start is subtracted from OD-end. Five replicates are used for each sample. These data result in a number that can be compared with the reference antibiotic amphotericin B.

Antiviral and cytotoxic screenings

The new compounds were screened at the Rega Instute, Minderbroederstraat 10, 3000 Leuven, Belgium against various pathogenic viruses such as the human immunodeficiency virus (HIV), herpes simplex virus (HSV), vaccinia virus (VV), the varicella zoster virus (VZV) and the human cytomegalovirus (CMV). For all viruses, except for CMV, the EC_{50} (effective compound concentration required to inhibit HIV-induced cytopathicity in human CEM cell cultures, HSV- and VV-induced cytopathicity in human embryo fibroblast E_6SM cell cultures, and VZV-induced plaque formation in human embryonic lung HEL cell cultures by 50%.) was determined. For determination of antiviral activity, expressed in IC_{50}, against CMV, human embryonic lung fibroblast (HEL) cells grown in 96-well microplates were infected with 20 PFU virus/well. After 2 hours of incubation at 37°C, the infected cells were replenished with 0.1 ml of medium containing serial dilutions of the test compound. On day 7 the plaques were counted microscopically after staining the cells with Giemsa's solution. The minimum antiviral concentration was expressed as the dose required to inhibit virus-induced plaque formation by 50%.

The compounds were also tested at the Rega Institute for anti-tumour activity via the inhibitory effects on the proliferation of murine leukemia cells (L1210/0), murine mammary carcinoma cells (FM3A), human T-lymphocyte cells (Molt4/C8, CEM/0) and human cervix carcinoma cells (HeLa), and are expressed in IC_{50}.

Cytotoxicity measurements were based upon the inhibition of HEL cell growth: HEL cells were seeded at a rate of 3 x 10^3 cells per well into 96-well microplates and allowed to proliferate for 24 hours in Eagle's minimum essential medium (MEM) containing various concentrations of the test compound. After 3 days incubation at 37°C, when the cell monolayer was 70% confluent, the cell number was determined with the Coulter counter (Beckman). The minimum cytotoxic concentration was defined as the concentration required to reduce cell growth by 50%.

Isolation, purification and structure elucidation

Equipment and materials

Separations were carried out with a straight phase column (Econosphere®, Alltech; silica 10µ, 150 x 22 mm) on an automated Gilson preparative HPLC system (322 pump) with UV-VIS detection (UV-VIS 156) and refractive index detection (RI detector 133). The samples were injected automatically (sampling injector 231 XL) and fractions were collected with an automated fraction collector (fraction collector 202).

The Rf values were determined via TLC on Merck silica gel (60F254, 0.25 mm thickness) and are not corrected.

Preparative TLC separations were carried out on Merck silica gel 60 (PLC plates 20 x 20 cm; 0.5 mm or PLC plates 20 x 20 cm; 2 mm). All solvents used were obtained from Acros Organics.

Chromatographic analyses were carried out with a reversed phase column (Ultrasphere, Beckman; (RP18; 5µm, 150 x 4.6 mm), a Waters 2690 HPLC with photodiode Array Detector (Waters 996) and mass detector (Q-TOF 2 Micromass).

Infrared spectra were taken on a Perkin-Elmer Spectrum GX 255 FT-IR spectrophotometer with 4^{-1} cm resolution.

MS spectra were taken on the same Q-TOF 2 (Micromass) in positive electrospray ionization mode. The capillary voltage was set at 2.8kV, the cone voltage at 45V, a collision energy of 10 eV, the MCP at 2250V, the source temperature at 120°C and the desolvatation temperature at 200°C, if not indicated otherwise.

^{1}H- and ^{13}C – NMR spectra were taken on a Brucker AM-500 and the chemical shifts (δ) are expressed in ppm and the coupling constants (J) in Hz.

The fragmentation of the parent ion ($M+H^+$) was studied via MS/MS analysis on the Q-TOF2 (Micromass) in positive electro spray ionization mode with a capillary voltage of 3kV, the cone voltage at 60V and the collision energy at 36V. The rest of the parameters were the same as mentioned above. The resulting mass spectrum was submitted to the MaxEnt3 algorithm (MassLynx, Micromass) and submitted to automatic peptide sequencing (MassLynx, Micromass) by adding a molecular weight of 18 to the parent ion in order to compensate for the cyclic nature of the peptides.

The peptide KPE01001001/0.1.0

The gram negative rod shape bacteria are incubated with triptic soy agar (5g) and yeast extract (2.5g) in 500ml of water at 37°C for a minimum of 48 hours so that fermentation starts and that the bacteria go to the log phase in order to obtain the secondary metabolites.

The suspension is then filter-sterilized and evaporated. The remaining solid material is redissolved in 50ml of water and extracted 3 times with 25ml of ethyl acetate. The organic layer is combined and concentrated via a rotavapor.

The extract is submitted to preparative HPLC using a Beckman Ultraprep RP-18 column (particle size 10µm) (150 x 21.2mm). Detection was done with a UV detector set at wavelength of 210mm. The eluent used was acetonitrile and water (ratio 75/25; flow 20ml/min). The total length of one run is 21 minutes. The peak with retention time 12.95 minutes was collected and the solvents were stripped off under reduced pressure using a rotavapor. The remaining solid white residue is the cyclic peptide KPE01001001/0.1.0.

The purity of the peptide was checked by analytical HPLC, using acetonitrile and water (ratio 75/25; flow: 1ml/min) as eluent. Detection was done with a UV detector set at a wavelength of 210nm and mass detector set at SIM of 1125.5, retention time was 7.27 minutes.

Rf value: 0.21 (solvent: CH_2Cl_2/MeOH (90/10))

The test with ninhydrine showed no colouring, meaning that the compound is cyclic and contains no free amines.

Molecular weight and formula: 1124.7 $C_{52}H_{92}N_{12}O_{15}$

IR (KBr): 3318.2; 2959.0; 2929.7; 2872.5; 1746.5; 1652.7; 1534.3; 1466.0; 1385.9; 1367.8; 1281.0; 1233.3; 1171.2; 1141.7; 1066.4; 960.0; 920.1; 668.5

The fragmentation of the parent peak ion (1125.5; $M+H^+$) in MS/MS mode, followed by peptide sequencing on the resulting spectrum is presented in **Figure 1.**

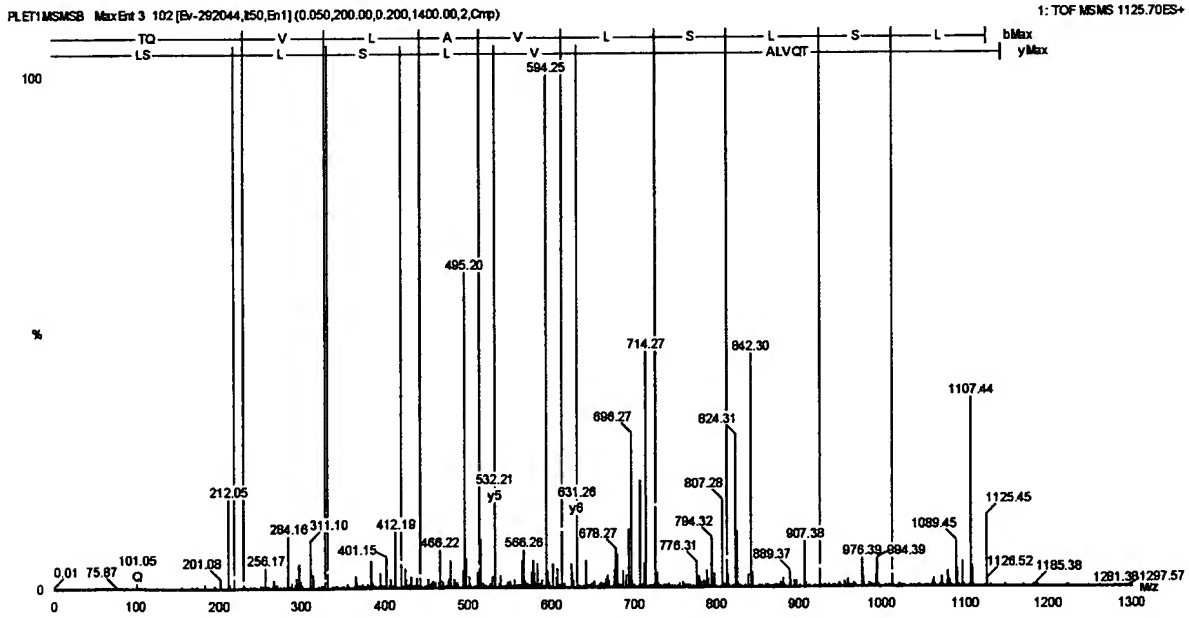

Figure 1: MS/MS spectra and peptide sequencing of KPE01001001/0.1.0

^{1}H MR (500 MHz, $CDCl_3$): 8.75 (1H, bs), 8.56 (1H, bs), 8.38 (1H, bs), 8.12 (2H, bs), 8.08 (1H, bs), 7.79 (1H, bs), 7.67 (1H, bs), 6.70 (1H, bs), 6.50 (1H, ,d, J=9.72), 6.28 (1H, s), 5.33 (2H, m), 4.87 (1H, bs), 4.60 (2H, m), 4.48 (1H, bs), 4.25 (1H, bs), 4.13 (3H, m), 4.00-3.85 (m), 3.80 (2H, m), 3.45 (1H, m), 2.50 (2H, m), 2.40 (2H, m), 2.19 (3H, d, J=2.83) 2.10-1.90 (m), 1.80-1.70 (m), 1.65-1.45 (m), 1.40-1.20 (m), 1.00-0.80 (36H, m).

^{13}C-NMR (125 MHz, $CDCl_3$): 176.07; 175.33; 174.95; 174.51;174.22; 173.99; 173.27; 172.98; 171.14; 168.46; 69.815; 69.661; 66.140; 64.934; 63.792; 61.502; 59.121; 56.945; 56.803; 56.215; 54.865; 53.815; 31.861; 29.607;29.363; 29.329; 26.125; 25.029; 24.745; 23.322; 23.083; 22.969; 22.720; 21.659; 21.583; 21.285; 20.533; 12.299; 18.460; 16.019; 14.196; 11.992.

Out of the combined analytical results, we concluded that KPE01001001/0.1.0 is a cyclic peptide that consists of 11 amino acids with a molecular weight of 1124.7 and cyclo-[TQVLAVLSLSL] as a sequence.

The peptide KPE01001007/1.3

The gram negative rod shaped bacteria is incubated with tryptic soy agar (5g) and yeast extract (2.5g) in 500ml of water at 37°C for a minimum of 48 hours so that fermentation starts and that the bacteria go to the log phase in order to obtain the secondary metabolites.

The suspension is then filter-sterilized and evaporated. The remaining solid material is redissolved in 50ml of water and extracted 3 times with 25ml of ethyl acetate. The organic layer is combined and concentrated via a rotavapor.

The extract is submitted to preparative HPLC using a Beckman Ultraprep RP-18 column (particle size 10µm) (150 x 21.2mm). Detection was done with a UV detector set at a wavelength of 210nm. The eluent used was acetonitrile and water (ratio 75/25), flow 20ml/min. The total length of one run is 21 minutes. The peak with retention time 15.3-17.7 minutes was collected and the solvents were stripped off under reduced pressure using a rotavapor. The remaining solid white residue is the cyclic peptide KPE01001007/1.3.

The purity of the peptide was checked by analytical HPLC, using acetonitrile and water (ratio: 75/25; flow 1ml/min) as eluent. Detection was done with a UV detector set at a wavelength of 210nm and mass detector set at SIM of 1125.5.

Rf value: 0.31 (solvent: Acetone/methanol (98/2))

The test with ninhydrine showed positive colouring; the mass spectrum indicates that this compound is cyclic in nature. Hence a free amino group is present in the side chain.

Molecular weight and formula: 1124.7 $C_{53}H_{96}N_{12}O_{14}$

IR (KBr): 3314.3; 2958.6; 2930.9; 2871.9; 1747.2; 1658.9; 1536.4; 1466.7; 1386.0; 1367.5; 1325.8; 1287.7; 1219.5; 1146.8; 1066.3; 959.6; 920.8; 868.6.

The fragmentation of the parent peak ion (1125.5; $M+H^+$) in MS/MS mode, followed by peptide sequencing on the resulting spectrum is presented in **Figure 2**.

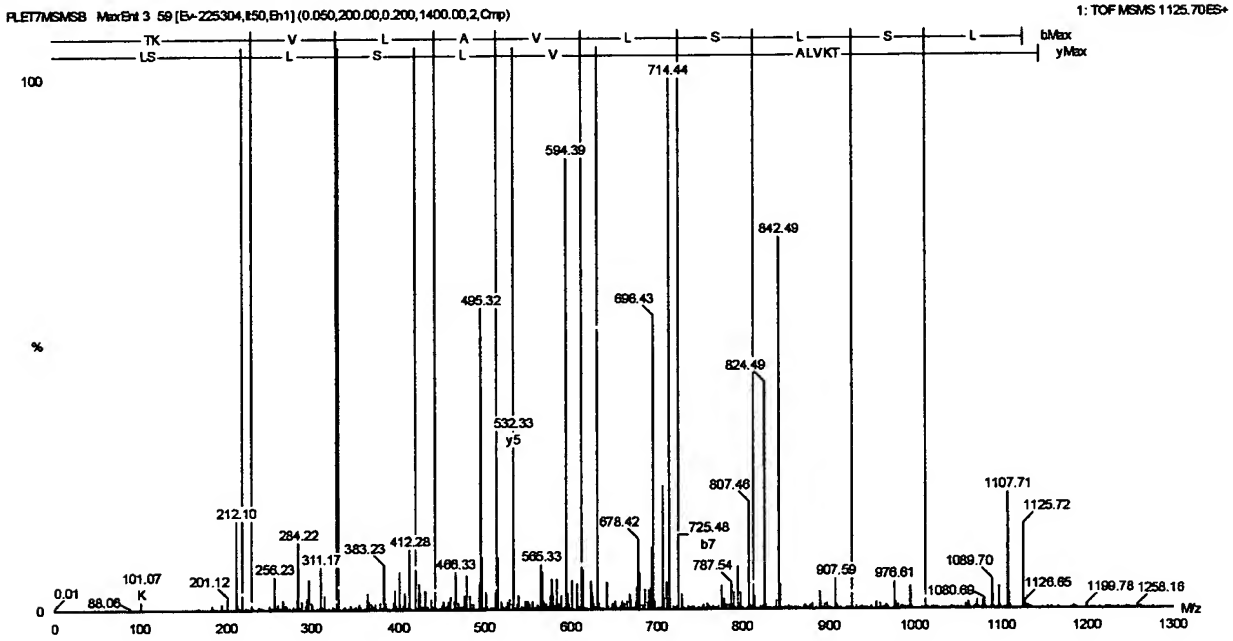

Figure 2: MS/MS spectrum and peptide sequencing of KPE01001007/1.3

^{1}H-NMR (500 MHz, $CDCl_3$): 9.00 (1H, bs), 8.61 (1H, bs), 8.44 (3H, bs), 8.10 (1H, bs), 8.07 (1H, bs), 7.11 (1H, bs), 6.88 (1H, bs), 6.49 (1H, bs), 6.25 (1H, bs), 5.23 (1H, bs), 4.84 (1H, bs), 4.61 (2H, bs), 4.27 (1H, bs), 4.15-3.70 (m), 2.17 (3H, d, J=3.27), 2.15-2.00 (m), 1.90-1.40 (m), 1.24 (3H, d, J=3.61), 1.05-0.80 (m).

^{13}C-NMR (125 MHz, $CDCl_3$): 177.12; 176.79; 176.02; 175.94; 174.79; 174.58; 173.81; 172.59; 172.06; 169.36; 70.41; 65.84; 61.92; 61.75; 59.48; 57.15; 56.51; 55.54; 54.48; 54.17; 53.56; 52.94; 41.27; 38.48; 38.05; 36.52; 35.94; 32.04; 31.20; 29.76; 29.53;29.50; 26.37; 24.85; 23.81; 23.48; 22.89; 22.83; 22.02; 21.72; 21.33; 20.45; 19.24; 18.55; 16.04; 14.26; 12.02.

Out of the combined analytical results, we concluded that KPE01001007/1.3 is a cyclic peptide that consists of 11 amino acids with a molecular weight of 1124.7 and cyclo-[TKVLAVLSLSL] as a sequence.

The peptide KPE01001006/1.0

The gram negative rod shaped bacteria is incubated with tryptic soy agar (5g) and yeast extract (2.5g) in 500ml of water at 37°C for a minimum of 48 hours so that fermentation starts and that the bacteria go to the log phase in order to obtain the secondary metabolites.

The suspension is then filter-sterilized and evaporated. The remaining solid material is redissolved in 50ml of water, acidified with phosphoric acid to pH = 2, and

extracted 3 times with 25ml of ethylacetate. The organic layer is combined and concentrated via a rotavapor.

The extracts are submitted to preparative TLC on silica using acetone and MeOH (ratio 90/10) as a solvent. The red fluorescent band (UV 365 nm) between 0.4 (Rf) and 0.6 (Rf) containing the compound was taken off and washed with the eluent. The remaining residue after evaporation was submitted again to preparative TLC on silica using acetone and MeOH (ratio 95/5) as a solvent. The broad red fluorescent band between 0.1 (Rf) and 0.5 (Rf) containing the compound was taken of and washed with the eluent. Evaportion of the solvent yields the compound.

The purity of the peptide was checked by analytical RP-HPLC using acetonitrile and water (ratio 75/25; flow 1ml/min) as eluent. Detection was done with a UV detector set at a wavelength of 210 nm and a mass detector with SIM at 1126.5. The retention time was 2 minutes.

Rf value: 0.5 (broad band) (solvent: acetone/methanol (90/10))

The test with ninhydrine showed no colouring, meaning that the compound is cyclic and contains no free amines.
Molecular weight and formula: 1125.7 $C_{52}H_{91}N_{11}O_{16}$

The fragmentation of the parent ion (1126.5; $M+H^+$) in MS/MS mode, followed by peptide sequencing on the remaining spectrum is presented in **Figure 3.**

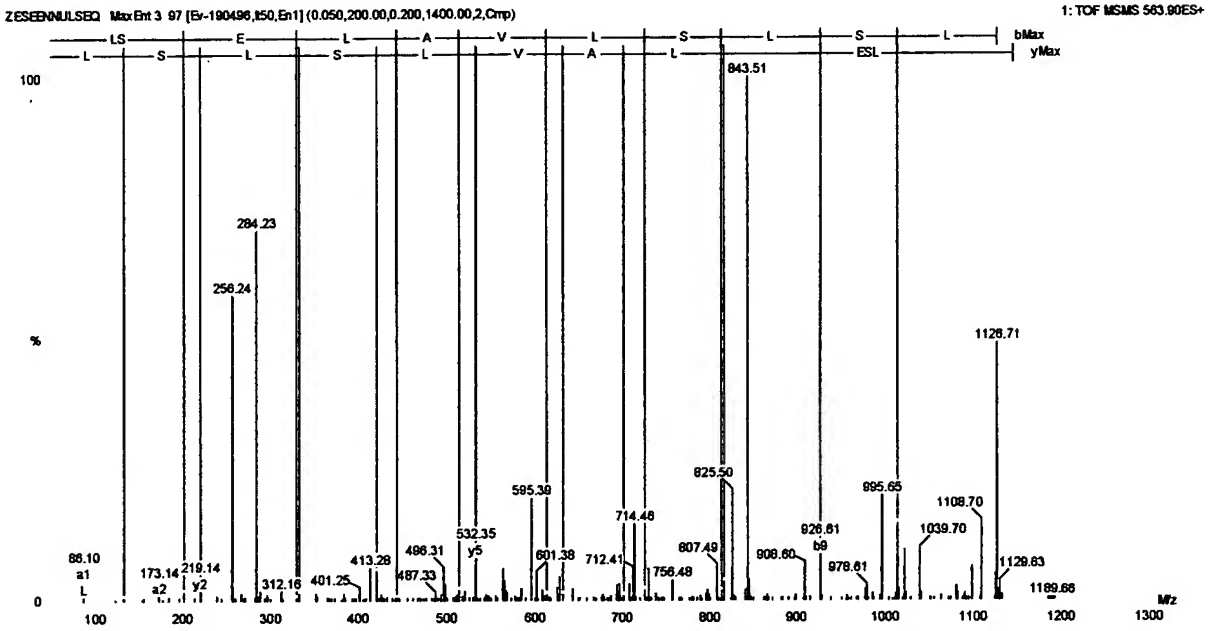

Figure 3: MS/MS spectrum and peptide sequencing of KPE01001006/1.0

Out of the combined results, we concluded that KPE01001006/1.0 is a cyclic peptide that consists of 11 amino acids with a molecular weight of 1125.7 and cyclo-[LSELAVLSLSL] as a sequence.

RESULTS AND DISCUSSION

During the broad antimicrobial screening of several crude extracts that were obtained after the extraction with ethyl acetate of fermentation mixtures derived from different gram negative bacteria living in symbiosis with amphibians, 3 interesting mixtures with antimicrobial properties were identified.

After purification of each crude extract by means of chromatographic techniques, one pure compound was isolated from each extract. The 3 molecules, coded KPE01001001/0.1.0, KPE01001007/1.3 and KPE01001006/1.0, were screened against several micro-organisms and cell line cultures in order to study the bio-active properties.

The results of the antiviral screenings are presented in Table 1.

Table 1: Antiviral activity of the cyclic undecapeptides

Compound	EC_{50}(µg/ml)[a]							IC_{50} (µg/ml)[b]	
	HIV-1 (III_B) (CEM)	HIV-2 (ROD) (CEM)	HSV-1 (KOS) (E_6SM)	HSV-2 (G) (E_6SM)	VV (E_6SM)	VZV (HEL)		CMV	
						OKA	YS	AD-169 Strain	Davis strain
KPE01001001/0.1.0	7	17	>16	>16	>16	3	3	8	>5
KPE01001007/1.3	7	>4	>16	>16	>16	ND	ND	ND	ND
KPE01001006/1.0	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] 50% effective concentration or compound concentration required to inhibit HIV-induced cytopathicity in human CEM cell cultures, HSV- and VV-induced cytopathicity in human embryo fibroblast E_6SM cell cultures, and VZV-induced plaque formation in human embryonic lung HEL cell cultures by 50%

[b] Inhibitory concentration required to reduce virus plaque formation by 50%. Virus input was 100 plaque forming units (PFU)

ND: not determined yet

A clear antiviral activity, more specific an activity against HIV1 and VZV, can be observed for KPE01001001/0.1.0 and for KPE01001007/1.3. For KPE01001006/1.0 no antiviral screening results are available yet.

In another High Throughput Screening, the antibiotic properties of the 3 isolates were evaluated **(Table 2)**. KPE01001001/0.1.0 and KPE01001007/1.3 have good antibacterial properties against gram positive bacteria, including resistant strains. Also possible antimycotic activities were studied **(Table 3)**. KPE01001006/1.0 showed good activity against *C. albicans* and against *C. neoformans.*

In addition to the antimicrobial effects, the molecules were also tested and evaluated for cytotoxic effects **(Table 4)**. From the cytostatic studies can be concluded that compound KPE01001001/0.1.0 and KPE01001007/1.3 showed cytostatic activity. Results for KPE01001006/1.0 are not available as of yet.

Table 2: Antibacterial activity

Compound	MIC in ppm (µg/ml)						
	E. faecalis ATTC 29212 LMG 8222	VRE ATCC 700221	*S. aureus* ATTC 29213 LMG10147	MRSA ATCC 33591 LMG1621 7	*P. aeruginosa* ATCC 27853 LMG6395	*S. typhimurium* ATTC700408 LMG16217	*C. perfringens* ATCC 13124
Positive Control	2.5a	>10a	1.0a	1.0a	1.0b	0.5b	0.5a
KPE01001001/0.1.0	6.25	6.25	6.25	6.25	>25	>25	6.25
KPE01001007/1.3	6.25	6.25	12.5	12.5	>25	>25	6.25
KPE01001006/1.0	>25	>25	>25	>25	>25	>25	>25

a: vancomycin b: gentamycin

Table 3: Antifungal activity

Compound	MIC in ppm (µg/ml)			
	Candida albicans IHEM 10284 – ATCC 24433	*Cryptococcus neofromans* IHEM 9558 ATCC 90112	*T. mentagrophytes* IHEM 10342 ATCC 9533	*Microsporum gypseum* IHEM 3999 – ATCC 14683
Positive Control (amphotericin B)	0.75	0.75	5.0	5.0
KPE01001001/0.1.0	>25	ND	>25	>25
KPE01001007/1.3	>25	ND	>25	>25
KPE01001006/1.0	<12.5	<12.5	>25	>25

ND = not determined

Table 4: Antitumour activity

Compound	IC_{50} (µg/ml)[a]				
	L1210/0	FM3A/0	Molt4/C8	CEM/0	HeLa
KPE01001001/0.1.0	17±2	16±	17±0	17±0	ND
KPE01001007/1.3	16±1	18±1	8.4±6.1	15±1	ND
KPE01001006/1.0	ND	ND	ND	ND	ND

[a] 50% inhibitory concentration

The bio-active molecules were submitted to several spectroscopic analyses in order to reveal the structure of these new isolates. Peptide sequencing by means of a MaxEnt3 processed the MS/MS spectrum for each molecule, indicated that the 3 molecules share a similar structure; they are all cyclic undecapeptides. These findings were confirmed by ^{1}H and ^{13}C NMR and FT-IR studies. The molecular structures and molecular weights are presented in **Table 5**.

Table 5: Molecular structures

Molecule	Structure	MW
KPE01001001/0.1.0	Cyclo-[TQVLAVLSLSL]	1124.7
KPE01001007/1.3	Cyclo-[TKVLAVLSLSL]	1124.7
KPE01001006/1.0	Cyclo-[LSELAVLSLSL]	1125.7

More detailed NMR experiments need to be done to see whether the molecules are cyclic peptides as such, or whether they are cyclic retro-peptides. In addition, hydrolysis followed by derivatisation with the Marfey's reagent has to be carried out in order to determine the absolute configuration of the different amino acids.

Closer study of the structure shows that the 3 molecules are built up from a part which is identical i.e. LAVLSLSL and a part which is unique for each molecule i.e. TQV for KPE01001001/0.1.0, TKV for KPE01001007/1.3 and LSE for KPE01001006/1.0. This unique part for each molecule is most probably what causes the difference in biological activity.

CONCLUSION

The 3 new studied molecules are isolated and purified secondary metabolites obtained from 3 different bacteria that live in symbiosis on the skin of amphibians. Structure elucidation by means of mass spectroscopy and NMR indicated that the 3 molecules are cyclic undecapeptides. A part of the sequence is identical (LAVLSLSL), another part is different and unique for each molecule (TQV for KPE01001001/0.1.0, TKV for KPE01001007/1.3 and LSE for KPE01001006/1.0).

This different part in the sequence causes a difference biological activity; KPE01001001/0.1.0 and KPE01001007/1.3 have a good and selective activity against gram positive bacteria, including resistant strains and on viruses such as HIV. They do not show significant activity against fungi.

On the other hand KPE01001006/1.0 has a good and selective activity against *Candida albicans* and *Cryptococcus neofromans*, but not against bacteria. Further studies need to be done to determine the absolute configuration of the amino acids and whether these molecules are peptides as such or retro-peptides.

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Cc: 'Raustin \(E-mail\)' <raustin@plethodon.com>
Date: Monday, August 19, 2002 05:19 am
Subject: RE: a new molecule

SUPER!!!!!

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<benedikt.sas@kemi To: <CENelson@Kemin.com>
npharma.be> cc: "'Raustin \(E-mail\)'" <raustin@plethodon.com>
Subject: RE: a new molecule
08/19/02 03:01 AM
Please respond to
benedikt.sas

even better now:

we have now another fraction (KPE01001013.1.4.1) that has an MIC of less than 1 PPM against specifically VRE!
This fraction still consists of several products, meaning that the MIC will be even better. Moreover, the fraction is highly selective and the best thing is that we have for the first time a fraction with an MIC<1 PPM allowing us to compete in the market. We will start working ASAP to determine the MW so that we can calculate the activity in nM.

Kind regards,

Bene

Dr. Benedikt Sas
General Manager Kemin Pharma
R&D Co-ordinator Kemin World-Wide

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From: CENelson@Kemin.com [ ]
Sent: 14 August 2002 00:05
To: benedikt.sas@keminpharma.be
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Subject: Re: a new molecule

Wow

Very cool news

Chris

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cc:
08/13/02 08:13 AM Subject: a new molecule
Please respond to
benedikt.sas

Dear both,

I want to inform you that we have found a new molecule : KPE01001014.1.6.
It has an exact MW of 323.1865 and a calculated possible brutoformula of
C11H27N6O3S. Most likely it is not a peptide. It seems not to be known
according some database searches.
The molecule has a high selective activity; momentarily we only found
activity against clostridium (MIC between 3 and 1.5 PPM).

Kind regards,

Bene

Dr. Benedikt Sas
General Manager Kemin Pharma
R&D Co-ordinator Kemin World-Wide

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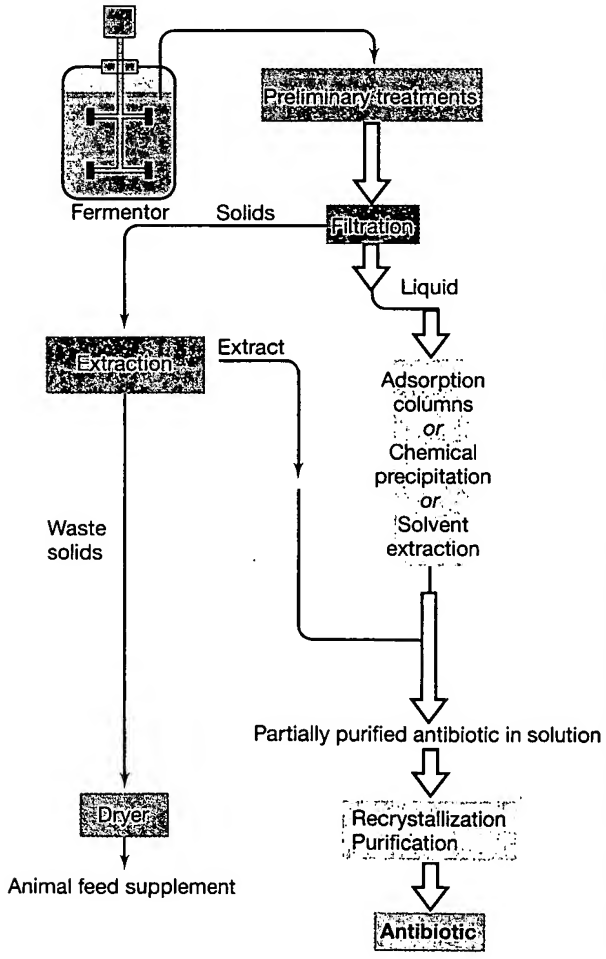
are then tested for antibiotic production by seeing whether they produce any diffusible materials that are inhibitory to the growth of test bacteria. The test bacteria used are selected from a variety of bacterial types but are chosen to be representative of, or related to, bacterial pathogens. The classical procedure for testing new microbial isolates for antibiotic production is the cross-streak method, first used by Fleming in his pioneering studies on penicillin (∞ the box, Microbiology and "Magic Bullets" in Chapter 20 and Figure 30.7*b*). Those isolates that show evidence of antibiotic production are then studied further to determine if the antibiotics they produce are new. Most of the isolates obtained produce *known* antibiotics, so the industrial microbiologist must quickly identify such organisms so that time and resources are not wasted in studying them. Once an organism producing a *new* antibiotic is discovered, the antibiotic is produced in sufficient amounts for structural analyses and then tested for toxicity and therapeutic activity in infected animals. Unfortunately, most new antibiotics *fail* these animal tests, but a few prove to be medically useful and are produced commercially. However, with estimates of the number of different antibiotics produced by species of the genus *Streptomyces* alone at over 100,000, research to discover new antibiotics occurs on a continuous bases.

Purification and Increased Yield

An antibiotic that is to be produced commercially must first be produced successfully in large-scale industrial fermentors. We discussed the general problem of scale-up in Section 30.4. The next challenge is the development of efficient purification methods. Because of the relatively small amounts of antibiotic present in the fermentation liquid, elaborate methods for extraction and purification of the antibiotic are necessary (Figure 30.8). If the antibiotic is soluble in an organic solvent, it may be relatively simple to purify it by extraction into a small volume of the solvent. If the antibiotic is not solvent-soluble, then it must be removed from the fermentation liquid by ad-



(a)

(b)

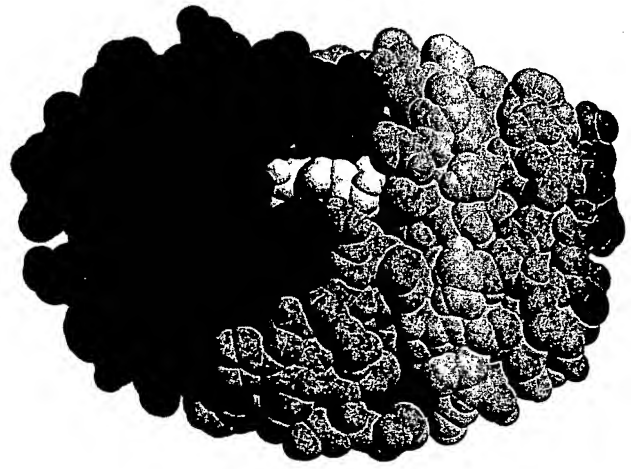
Figure 30.8 Purification of an antibiotic. (a) Overall process of extraction and purification. (b) Installation for the solvent extraction of an antibiotic from fermentation broth. Effective engineering is as important as microbiological factors in the successful production of an antibiotic.

vitro biological activity on different microorganisms, also using automated techniques. The automated synthesis and screening processes dramatically shorten drug discovery time and increase the number of new candidate drugs by 10 times or more each year.

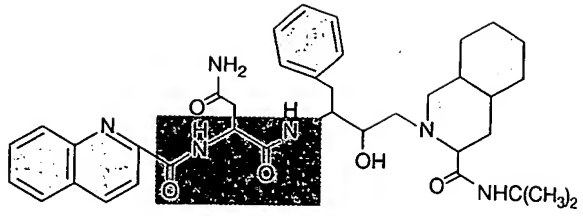
According to pharmaceutical industry estimates, about 7 million candidate compounds must be screened to yield a single useful clinical drug. As we have just discussed, this process starts with synthesis, isolation, and screening of candidate compounds. Screening involves *in vitro* tests used for antibiotic susceptibility (see Section 20.4). Drugs effective in the laboratory must then be tested for efficacy and toxicity in animals and finally in clinical trials in humans. Animal testing may take several years and multiple trials to ensure that the candidate drug is efficacious and safe for use. For the same reasons, clinical trials usually take years to complete for each drug. New drug discovery and development typically takes 10 years to 25 years before approval for clinical use. The cost of discovery and development is estimated to be about $500 million for each new approved drug.

Computerized Drug Design

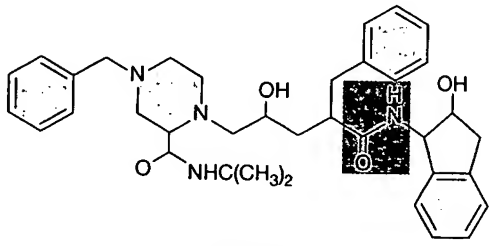
Truly novel antimicrobial compounds are much more difficult to identify than analogs of existing drugs because new antimicrobial compounds must work at unique sites in metabolism and biosynthesis, or be structurally dissimilar to existing compounds to avoid existing resistance. To find these new compounds, candidate drugs had to be isolated from natural sources and systematically screened for antimicrobial activity. However, recent advances in computer and structural graphics technology now make it possible to design a drug to interact with specific known microbial structures. Drug discovery can now begin at the computer, where new drugs can be rapidly "created" and "tested" for binding and toxicity in the computer environment at relatively low cost (Sections 30.4 and 26.8). One of the most dramatic recent successes in computer-directed drug design is the development of saquinavir, a protease inhibitor that is used to slow the growth of the human immunodeficiency virus (HIV) in infected individuals (Figure 20.27). HIV protease cleaves a virus-encoded precursor protein to produce the mature viral core and activate the reverse transcriptase enzyme necessary for replication (Section 16.14). Saquinavir was designed by computer to fit the active site of HIV protease, based on the known three-dimensional structure of the protease-substrate complex; it is a peptide analog that displaces the HIV precursor protein, inhibiting virus maturation and slowing its growth in the human host. A number of other computer-designed protease inhibitors like saquinavir are in use as chemotherapeutic drugs for the treatment of AIDS (Table 20.5, Figure 20.27, and Figure 26.38). As this example demonstrates, com-



(a)



Saquinavir



Indinavir

(b)

Figure 20.27 Computer-generated antiviral drugs. (a) The HIV protease homodimer. Individual polypeptide chains are shown in green and blue. A peptide (yellow) is bound by the catalytic site. This protease cleaves an HIV precursor protein, a necessary step in virus maturation (Section 16.14). Blocking of the protease site by the peptide shown inhibits precursor processing and HIV maturation. This structure is derived from information in the Protein Data Bank. (b) These anti-HIV drugs are peptide analogs that were designed by computer to block the active site of HIV protease. The areas highlighted in orange show the regions analogous to peptide bonds. Binding of these compounds by the HIV protease prevents HIV precursor processing and virus maturation. These compounds are representative of a class of therapeutic drugs known as non-nucleoside reverse transcriptase inhibitors (NNRTI). The concentration of these compounds in HIV-infected cells, coupled with their strong affinity for HIV reverse transcriptase, makes them very potent competitive inhibitors for the active sites of the transcriptase and prevents viral replication. These protease inhibitors are widely used for treatment of HIV infection (see Table 20.5 and Section 26.14).